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The regulation of reserve mobilization in cotyledons of lipid-rich *Cucumis sativus* L. seeds

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Abstract

Conflicting results in the literature regarding the possible regulatory role of the embryonic axis on reserve mobilization in lipid-rich seeds prompted this study on cucumber (*Cucumis sativus*) seed. Two existing models supporting control by the axis, namely the hormonal control- and source-sink relationship models, were tested by incubating detached cotyledons in plant hormone solutions and axes extracts as well as in the presence of artificial sinks. This approach was an attempt to alleviate any effect that axis removal might have on reserve mobilization in cotyledons measured in terms of changes in fresh and dry weight, respiratory capacity and isocitrate lyase activity. No clear evidence that lipid mobilization in cucumber seeds was controlled by the axis through either a hormonal- or a source-sink mechanism was obtained. However, axis removal prevented the natural shedding of the testa by germinating cucumber seeds resulting in clear differences between intact and detached cotyledons in terms of fresh and dry weight, respiratory and lipid metabolism. Moreover, weakening of the testa on detached cotyledons, by cutting longitudinally, only partially alleviated the inhibitory effect that axis removal had on these metabolic events while total removal alleviated this effect completely. The latter was more pronounced in an elevated oxygen atmosphere indicating that the testa played a more pronounced role in controlling the metabolism of cucumber cotyledons during the post-germinative seedling establishment phase than did the growing axis.

Keywords: Axis control; Cotyledons; *Cucumis sativus*; Lipid mobilization; Testa

1. Introduction

A hormonal mechanism for reserve mobilization was first described for monocotyledonous cereal grains, where the embryo regulates the synthesis of hydrolytic enzymes in the aleurone layer through the action of hormones (Yomo and Varner, 1973). Except for single reports on storage reserve mobilization in dicotyledonous oil rich rape (*Brassica napus*; Nykiforuk and Johnson-Flanagan, 2000; Chia et al., 2005), mung bean (*Vigna mungo*; Maki and Morohashi, 2002) and Indian bean (*Dolichos lablab*; Ramakrishna and Ramakrishna, 2006) seeds, *Arabidopsis thaliana* has become very popular as a model system for this type of research in recent times (Penfield et al., 2005). Pritchard et al. (2002) reported that germination and storage reserve mobilization are regulated independently in lipid-rich *Arabidopsis* seed while Penfield et al. (2006) maintained that regulation of lipid reserve mobilization during seed germination of *Arabidopsis* requires coordinate action by the embryo and surrounding endosperm. It is clear that conflicting results still exist even in the *Arabidopsis* model.

Literature reports on storage reserve mobilization in cucumber (*Cucumis sativus*) seeds are scarce and rather outdated. For example, more than two decades ago Davies and Chapman (1979a,b) reported that lipid breakdown is inhibited in excised cucumber cotyledons in the absence of the axis. Two alternative models originated in order to explain the hypothesis that food mobilization is under axial control in seeds of many dicotyledonous plants. The first involves a source–sink relationship that exists between the storage organs and axis during germination and early seedling development (Davies and Chapman, 1979a,b; Davies and Slack, 1981; Chapman and

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Davies, 1983). The second involves the production of a hormonal stimulus by the axis that initiates the development of optimal rates of hydrolytic enzyme activity in the storage organs (Nandi et al., 1995). The latter two hypotheses were challenged in this study.

The first hypothesis proposed that the growing axis simply acts as a sink, mobilizing hydrolyzed reserve products in the cotyledons by means of a concentration gradient (Davies and Chapman, 1979a,b; Davies and Slack, 1981; Chapman and Davies, 1983). In cucumber, this hypothesis was supported as axis excision resulted in an appreciable reduction in lipid mobilization in the cotyledons without affecting the development of lipolytic enzymes (Slack et al., 1977; Davies and Chapman, 1979a). Following axis excision, the authors maintained that an inverse correlation exists between the subsequent inhibition of lipid hydrolysis and the accumulation of reducing sugars and sucrose in the cotyledons irrespective of whether the axis was removed immediately after or 2–4 days after imbibition. This implies that the cucumber axis exerts its influence via a sink effect.

The second hypothesis received support from results published by a number of authors over the past three decades. It was postulated that, in seeds of dicotyledonous plants, cytokinins and probably also gibberellins are transported from the growing axis to the storage tissue to regulate reserve mobilization (Locker and Ilan, 1975; Ilan and Gepstain, 1981; De Klerk, 1986; Nandi et al., 1995). Mobilization of carbohydrate reserves is apparently affected by cytokinins, which enhance α-amylase activity in bean cotyledons (Gepstain and Ilan, 1970) while gibberellins act similarly on α-amylase production in peas (Varner et al., 1963). Exogenously applied cytokinins and gibberellins can both replace the influence of the embryonic axis on endo-β-mannanase production in lettuce seed endosperm, while ABA inhibits the production of this enzyme (Halmer and Bewley, 1979, 1982).

Besides the existing hypotheses on the regulation of reserve mobilization in dicotyledonous seeds, it has been shown that the intact testa inhibits O_2 uptake by the cotyledons (Slack et al., 1977). In the Cucurbitaceae the testa is naturally shed soon after germination by a peg of tissue which forms on the lower surface of the hypocotyl and which serves to lever off the integument (Witztum and Gersani, 1975). If the embryonic axis is removed, however, the peg does not form, the testa is not displaced and both lipid and protein degradation are inhibited (Davies and Chapman, 1979a). Removal of the inner layer of cells from squash cotyledons, that naturally remains after the seed coat is removed, also speeds up these early metabolic events in the cotyledons including the presence of isocitrate lyase (Rollo et al., 1972), a key enzyme involved in lipid breakdown (Breidenbach and Beevers, 1967).

This interesting mechanism of testa shedding in cucumber seeds, as well as the fact that a membrane, the perisperm, remains following removal of the seed coat, supplied the rationale for investigating the events that lead up to germination as well as subsequent seedling growth. Further, it is possible and even likely that no single reserve mobilization mechanism operates in all dicotyledonous seeds. In this study it was postulated that removal of the axis from cucumber seeds would provide a means to study its effect on the reserve mobilization process in cotyledons. The objective was also to seek one or more treatments capable of alleviating the effect of axis removal on cotyledonary metabolism in an attempt to find means to test the type of control, if any, by the axis. For this purpose, different treatments were applied including substitution of the embryonic axis with plant growth substances (Tarpley and Choinski, 1986) and the creation of artificial sinks. The postulate that lipid mobilization in the cotyledons is a post-germinative event was additionally investigated by using different metabolic inhibitors, while the possible regulating role of the testa was followed by manipulating the testa in different ways. It was envisaged that, from these approaches, different treatment methods would be identified that would assist in follow-up studies on the metabolic mechanisms of reserve mobilization in the cotyledons of cucumber seeds during germination and early seedling growth.

2. Materials and methods

2.1. Plant material

Cucumber seeds, cv. Special Rust Resistant, were purchased from a local seed merchant and stored in sealed containers at 4 °C in the dark at a relative humidity of 74%. Prior to treatments, seeds were removed from cold storage and maintained at room temperature for at least 24 h. Medicinal grade oxygen (O_2), nitrogen (N_2) and ethylene were purchased from Fedgas, South Africa. All other chemicals used were from Merck (Germany) and were of the highest purity available.

2.2. Seed germination

Triplicate batches of 20 seeds were germinated on a single layer of filter paper (Schleicher & Schuell, No.595) in 9-cm-diameter glass Petri dishes and moistened with 8 cm^3 distilled water or relevant test solutions. This volume was previously confirmed to be sufficient to sustain germination and early seedling growth for at least 72 h. Incubation of the seeds was in a Labcon Model L TIE growth cabinet at 30 °C ± 0.5 °C in the dark, previously confirmed to be the optimum germination temperature for this cultivar. The time course of axis emergence was determined at set intervals. A seed from which the radicle protruded through the testa was taken as germinated.

2.3. Removal of axes and testae

Certain treatments required the removal of either the axis or testa or both at different incubation intervals after completion of germination (24 h). The axis was removed from the cotyledons using a sharp razor blade, taking care that no axis tissue remained attached to the cotyledons.

2.4. Extraction of isocitrate lyase (ICL; EC 4.1.3.1) and determination of enzyme activity

Where applicable, seed axes and cotyledons were separated before enzyme extraction. Twenty cotyledons per replicate were

homogenized using a mortar and pestle in pre-cooled (2 °C) extraction buffer at a ratio of 5 cm^3 g^{-1} fresh weight. The K-phosphate extraction buffer, with a final concentration of 100 mM, was prepared using KH_2PO_4 and K_2HPO_4 in specific ratios in order to obtain a pH of 7.5. The buffer additionally contained 400 mM sucrose, 0.1 mM $MgCl_2$ and 1 mM EDTA (Dixon and Kornberg, 1959). Prior to extraction, 2 mM Phenylmethylsulfonyl fluoride (PMSF, dissolved in 100% ethanol) and 0.1% (v/v) Triton X-100 were added to the medium. The homogenate was centrifuged in a Hettich micro-rapid K-bench centrifuge at maximum speed (12000 rpm) for 10 min at 2 °C. Enzyme activity was determined in the supernatant in a final volume of 1 cm^3 using disposable cuvettes.

The assay mixture consisted of 66 mM K-phosphate buffer (pH 6.85), 5 mM $MgCl_2$, 2 mM L-cysteine, 3.5 mM phenylhydrazone and 1.8 mM isocitrate. The crude enzyme protein concentration used during the assays was 0.1–0.2 mg and the rate of change in absorbance was followed at 324 nm at 25 °C using a temperature controlled Hitachi U-2000 Pye Unicam spectrophotometer. The reaction was started with the addition of isocitrate (Ford et al., 1976) and the A_{324} was determined for at least 7 min (Dixon and Kornberg, 1959). All assays were linear with respect to time and enzyme concentration.

The effect of certain metabolic inhibitors on the *in vivo* activity of ICL in intact cotyledons was determined in extracts prepared from seeds incubated from 24 h to 48 h in the inhibitor solutions. Additionally, the effect of metabolic inhibitors on the *in vitro* activity of ICL was determined as follows: After ICL activity was obtained in a cotyledon extract from 48 h intact seeds imbibed in water only, the inhibitor solution was added to the spectrophotometer cuvette at the required concentration and its effect on the *in vitro* activity followed until a linear response was obtained. Inhibition of both the *in vivo* and *in vitro* activities of ICL by these inhibitors was expressed as % inhibition in relation to control activities.

2.5. Determination of protein content of seed material

The total protein content in the extracts used for measuring ICL activities was determined using the dye-binding technique of Bradford (1976). Ten µl of standard or sample and 40 µl Biorad dye reagent was added to microplate wells in a final volume of 200 µl made up with double distilled water. The absorbance of the assays was determined at 595 nm using a Bio-Rad (Model 3550) microplate reader. Bovine gamma-globulin (0,4 mg cm^{-3}) was used as standard. The means of four assays were used to calculate the protein content of the samples.

2.6. Determination of the respiration rate and RQ values of cucumber cotyledons

The rate of O_2 consumption (respiration rate) by control, treated and detached cotyledons were determined by means of constant pressure manometry, using a submersible differential Gilson respirometer. Five to 10 cotyledons, depending on the treatment, were placed in Warburg reaction vessels containing 1 cm^3 incubation medium (distilled H_2O or different growth hormone solutions). The rate of both net gas exchange and O_2 consumption by the cotyledons were determined and gas exchange values were corrected according to the method of Gregory and Purvis (1965). Carbon dioxide liberation and RQ values were calculated.

2.7. Treatment of seeds or seed parts

2.7.1. Incubation of detached cotyledons in the presence of intact axes, an axis extract and artificial sinks

The hypothesis that natural growth substances are synthesized by the axis or root tip and subsequently transported to the cotyledons to control reserve mobilization (Bewley and Black, 1994) was tested using two different approaches. Firstly, incubation of detached cotyledons in (i) 5 cm^3 of an aqueous axes extract and (ii) in the presence of 20 isolated intact axes for 48 h, where-after the rates of O_2 consumption by, and ICL activity in, the detached cotyledons were determined. Seventy two-h-old axes were chosen because, at this time lipid metabolism was highly active. In the second approach, different commercially available plant hormones were used for incubation of detached cotyledons. The different plant hormones and the concentrations used were: IAA (20 mg/L; 114 µM), 2,4-D (20 mg/L; 90 µM), kinetin (20 mg/L; 92 µM), Benzyladenine purine (BAP; 20 mg/L; 88 µM), GA_3 (100 mg/L; 260 µM), ethrel (300 µ1/L) and ethylene (200 µl/L).

The hypothesis that reserve mobilization in dicotyledonous seeds is controlled by a source–sink relationship (Davies and Slack, 1981; Chapman and Davies, 1983) was tested by incubating detached cotyledons in the presence of artificial sinks in two different ways. Detached cotyledons were incubated in an inverted position, with the side from which the axes were removed in contact with either filter paper moistened with 5 cm^3 distilled H_2O or partially submerged in a thin layer of 1% (w/v) aqueous agar medium, in a Petri dish.

2.7.2. Treatment with test solutions

Seeds and seed parts (excised axes or cotyledons) were placed in Petri dishes, after completion of germination (24 h), on filter paper moistened with 5 cm^3 distilled water or test solution (e.g. hormonal solutions or metabolic inhibitors). Incubation of detached cotyledons in volatile solutions of KCN, ethrel or ethylene was performed in sealed Schott bottles on filter paper moistened with 5 cm^3 solution. In the case of ethylene, calculated volumes of pure ethylene (v/v) were injected through the rubber septa to deliver the required concentrations. All treatments were carried out in triplicate.

2.7.3. Weakening of the testa

The possible restraining effect of the testa on reserve mobilization was investigated by weakening the testa in two ways. In addition to the cut edge on the side of the seed where the axis was removed, the testa was further weakened by either cutting it horizontally on the distal end from the axis or by cutting it longitudinally two thirds down from the distal end front to back and on both sides along the seam of the testa. This also removed the thin perisperm which was mostly attached to

the testa. The possible combined effect of the axis (sink) and the mechanical resistance of the testa were tested by incubating detached cotyledons with longitudinally weakened testae on 1% (w/v) agar in Petri dishes that served as an artificial sink.

2.7.4. Exposure of detached cotyledons to different oxygen atmospheres

When treated seeds or detached cotyledons, with the testa either intact or totally removed, were incubated under anaerobic or aerobic conditions, the material was placed on one layer of filter paper in 600 cm^3 Schott reagent bottles, moistened with 5 cm^3 test solution, sealed with a rubber septum and flushed for at least 30 min with either pure N_2 or O_2 before incubation at 30 °C in the dark for up to 72 h. Where different O_2 concentrations were used, the bottles were first flushed with N_2 where-after calculated volumes of N_2 in the sealed bottles were displaced by pure O_2 through the rubber septa to deliver the required concentrations. In a control treatment, intact seeds were transferred to a sealed Schott bottle directly after germination (24 h) and flushed with pure N_2 for 30 min before incubating as described above.

3. Results

Germination of untreated control seeds was completed for the first seeds after 12 h of incubation and for all by 24 h (Fig. 1A). After an initial increase, the fresh mass of the cotyledons remained constant during germination and increased slightly upon completion of germination (Fig. 1B) while the fresh mass of the axes increased rapidly after completion of germination. The moisture content of both the axes and cotyledons showed a marked increase upon imbibition and reached a plateau during germination (Fig. 1C). However, when seedling growth commenced, the moisture content of both seed parts increased further with that in the axes being more pronounced after germination.

Removal of the axis from 24-h-incubated seeds clearly reduced its ability to grow in terms of length (Fig. 2A), fresh mass (Fig. 2B) and dry mass accumulation (Fig. 2C) over a 72 h incubation period, emphasizing its dependence on the cotyledons for growth. However, the moisture content (Fig. 2D) of intact and excised axes remained unchanged over the same period.

Removal of the axis also prevented an increase in both the fresh mass (Fig. 3A) and moisture content (Fig. 3C) of detached cotyledons during a 72 h post-germinative incubation period when compared to the intact control cotyledons. Changes in the dry mass (Fig. 3B) of detached cotyledons, however, paralleled that of the intact control cotyledons during the same period.

Subsequently, the effect of metabolic inhibitors on germination and seedling growth was investigated in an attempt to obtain information on the possible metabolic events preceding germination of cucumber seeds as well as to identify main post-germinative metabolic events. Both KCN and NaN_3, well known inhibitors of cytochrome oxidase in the mitochondria, completely inhibited germination at concentrations of 1 mM (Table 1). Inevitably, no axial growth was possible in these two

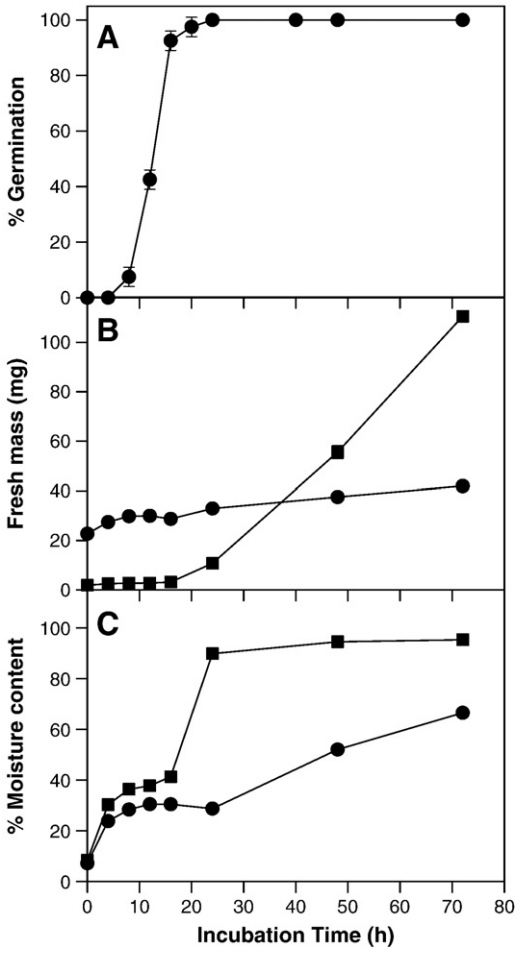

Fig. 1. Percentage germination of cucumber (*Cucumis sativus* L.) seeds (A) as well as fresh mass (B) and moisture content (C) of axes (■) and cotyledons (●) during the first 72 h of incubation.

cases. In contrast, all the other inhibitors applied (see Table 1 for modes of action) had no or only a slight effect on seed germination but, in most cases, inhibited post-germinative axial growth to varying degrees. The most potent inhibitors of axial growth were ARS, 2,4-DNP and SHAM, all well known inhibitors of mitochondrial respiration, CHX, an inhibitor of cytosolic protein synthesis and IA, an inhibitor of isocitrate lyase (ICL) activity in the glyoxysomes and succinate dehydrogenase activity in the mitochondrion. Chloramphenicol (CA), an effective inhibitor of organelle protein synthesis, had virtually no effect on seed germination but markedly inhibited post-germinative axial growth (Table 1).

In light of the fact that most of the metabolic inhibitors applied affected post-germinative axial growth more than germination itself, the effect of certain metabolic inhibitors on the activity of ICL, a key glyoxysomal enzyme involved in lipid breakdown during this period, was subsequently investigated. Moreover, it was also necessary to determine whether ICL was already present in the cotyledons on completion of germination or newly synthesized *in vivo* before lipid mobilization commenced. For this reason both the *in vivo* and *in vitro* activities of ICL were measured. All the specific metabolic inhibitors used almost completely inhibited the *in vivo* specific

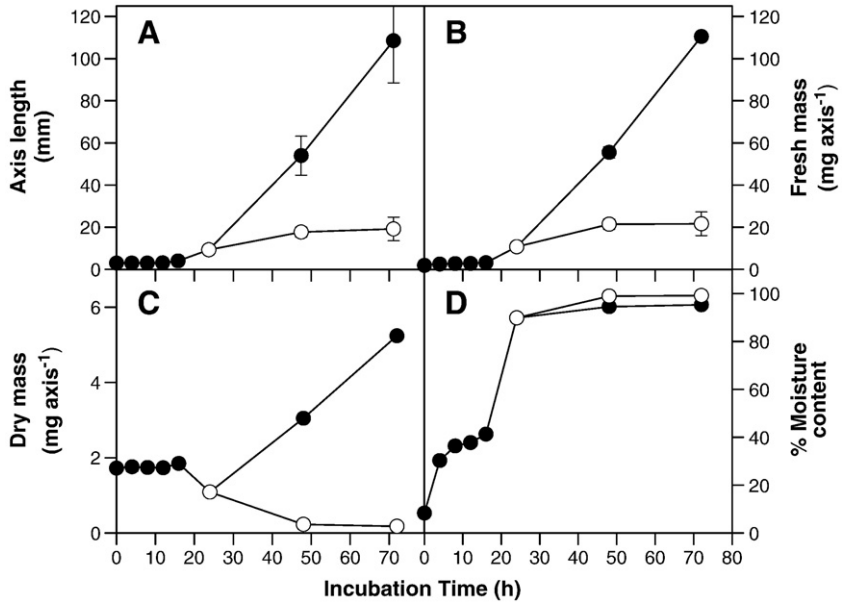

Fig. 2. Changes in length (A), fresh mass (B), dry mass (C) and moisture content (D) of intact cucumber (*Cucumis sativus* L.) axes (●) and axes detached from its cotyledons (○) at 24 h of incubation.

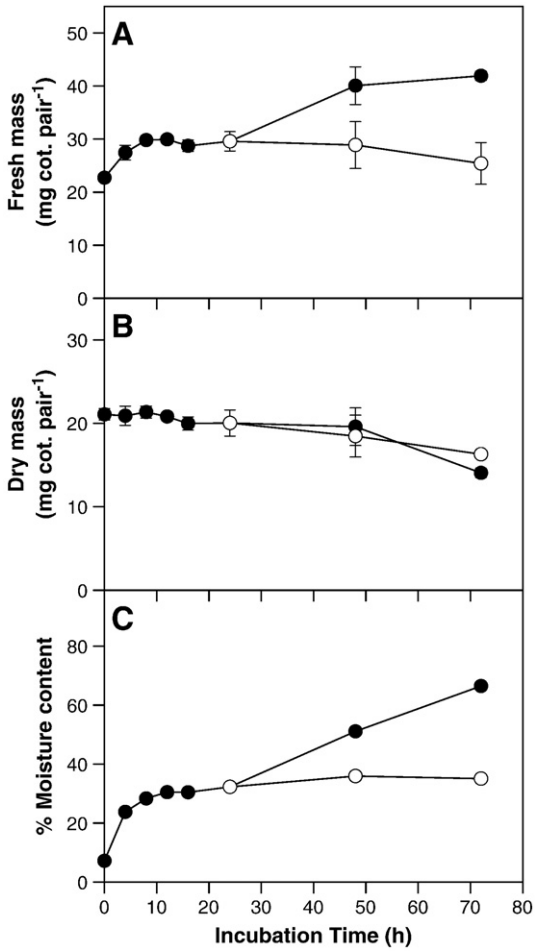

Fig. 3. Changes in the fresh mass (A), dry mass (B) and moisture content (C) of intact cucumber (*Cucumis sativus* L.) cotyledons (●) and detached cotyledons (○). Axes were removed at 24 h of incubation.

activity of ICL in the cotyledons (Table 2). Except for IA, a potent inhibitor of ICL, and to a lesser extent KCN, none of the other inhibitors affected the *in vitro* activity of ICL suggesting that only IA had a direct inhibitory effect on ICL activity while the rest probably affected other metabolic events that inevitably lead to the inhibition of the *in vivo* activity of ICL. This was also indicative of lipid mobilization being mainly a post-germinative event in cucumber cotyledons.

Subsequently the effect of axis removal on metabolic events in the cotyledons, as well as different treatments to alleviate this effect, was investigated using respiration rate, RQ values and ICL activity as parameters. The respiration rate (O_2 consumption) of control intact cotyledons (Table 3A) increased twenty fold during seedling establishment (24 h–72 h). Concomitantly, the RQ value significantly decreased during this period indicating a switch towards lipid metabolism ($RQ \leq 0.7$) and ICL activity increased almost threefold. Removal of the axis prevented an increase in both the respiration rate and the ICL activity in detached cotyledons while the RQ value did not decrease as much as when cotyledons and axes were still intact (Table 3A).

Additional manipulation treatments were used to determine if these could substitute for the possible controlling effect of the axis. Of all the externally supplied plant hormones, ethylene was the only one that stimulated the rate of O_2 consumption (Table 3B) but it was insignificant when compared to that of the intact control cotyledons (Table 3A). Moreover, the RQ values were not markedly affected by any of the plant hormones and none alleviated the inhibitory effect of axis removal on the specific activity of ICL in the detached cotyledons.

Replacement of the axis with filter paper or 1% (w/v) agar artificial sinks (Table 3C) failed to alleviate the effect of axis removal on either the respiration rate, including the RQ value, or the ICL activity. However, longitudinal weakening of the

Table 1
List of metabolic inhibitors, their possible inhibition sites and concentrations used during germination studies

Metabolic Inhibitors	Concentration (mM)	Important inhibition sites in plant tissues	% Germination after 72 h	Average axis length after 72 h (mm)
None (Control)			100±0	108.5±20.0
Potassium cyanide (KCN)	1	Cytochrome-c oxidase; Catalase; Electron transport in the chloroplast; Rubisco of some plants; Transport of glucose; Transport of succinic acid	3.30±2.4	0±0
Sodium azide (NaN_3)	1	Cytochrome-c oxidase; ATPase; Catalase; Initiation of protein synthesis; *In vivo* RNA synthesis	0±0	0±0
Sodium arsenate (ARS)	1	Mitochondrial respiration; Oxidative phosphorylation; Transport of phosphate	86.7±6.2	1.0±0
2,4-Dinitrophenol (2,4-DNP)	1	Uncoupler of oxidative phosphorylation; NADH dehydrogenase (Quinone)	91.7±2.4	1.0±0
Salicylhydroxamic acid (SHAM)	1	Alternative oxidase in plant mitochondria	85.0±4.1	75.0±3.1
	5		75.0±2.4	1.0±0
Itaconic acid (IA)	5	Isocitrate lyase (ICL); Succinate dehydrogenase (SDH)	93.3±2.4	35.0±2.4
Cycloheximide (CHX)	2	Cytosolic protein synthesis; Synthesis of Cytochrome oxidase; DNA synthesis; Methylation of r-RNA	85.0±7.1	50.0±3.3
	5		75.0±4.1	25.0±1.8
Chloramphenicol (CA)	2	Organelle protein synthesis; NADH-dehydrogenase (Ubiquinone)	91.7±4.7	50.0±3.3
	10		100±0	25.0±1.8

testa alleviated the negative effect of axis removal on O_2 consumption and RQ values to a large extent compared to other treatments (Table 3D). The respiration rate of detached cotyledons with longitudinally weakened testae was almost 50% of that of the intact control cotyledons (Table 3A). However, this treatment had no stimulatory effect on the activity of ICL in the detached cotyledons.

Incubating detached cotyledons in a 100% O_2 atmosphere alleviated the reducing effect axis removal had on both O_2 consumption and ICL activity to a large extent (Table 3E). When the testa was removed and detached cotyledons were incubated in air this trend was more accentuated than when the testa remained intact or seeds incubated in O_2. The RQ value, however, was much lower in the latter instance.

Total removal of the testa from intact seeds (Table 3F) resulted in a significantly higher respiration rate while the ICL activity paralleled that of intact seeds from which the testa was not removed (Table 3A). Incubation of intact seeds in nitrogen (Table 3F) reduced both the respiration rate and ICL activity to extremely low levels.

4. Discussion

Bewley and Black (1994) reported that storage reserves in cucumber cotyledons are mobilized and supplied to the developing embryo only after completion of germination and until the seedling becomes autotrophic. In this study a series of metabolic inhibitors with different action mechanisms were applied exogenously to germinating cucumber seeds to test this postulate. Incubation of whole seeds in KCN and NaN_3 solutions, both inhibitors of cytochrome oxidase in the mitochondria, prevented seeds from germinating indicating that mitochondrial activity in either the axes or cotyledons or both is essential for germination to proceed. Moreover, both KCN and NaN_3 effectively inhibited the *in vivo* specific activity of ICL in cucumber cotyledons, while it had little effect on the *in vitro* activity. This indicated that inhibitors of mitochondrial respiration most probably affected the close relationship between the mitochondria and the glyoxysomes (Mettler and Beevers, 1980) in such a way that it inevitably affected the

Table 2
Effect of different metabolic inhibitors on the *in vivo* and *in vitro* specific activity of ICL in 48 h cotyledon extracts

Incubation medium	Conc. (mM)	Specific activity of ICL after 48 h: (nmol min^{-1} mg $prot^{-1}$)		% Inhibition	
		in vivo	*in vitro*	*in vivo*	*in vitro*
Water (Control)	–	30.7±4.0	30.7±4.0	0%	0%
NaN_3	1 mM	4.9±0.8	30.7±1.7	84%	0%
KCN	2 mM	0.6±0.1	19.0±1.2	98%	38%
Itaconic acid (IA)	5 mM	1.9±0.2	0±0	94%	100%
Cycloheximide (CA)	5 mM	2.3±0.9	30.4±2.1	92%	1%

Table 3
The effect of axial removal and different treatments on O_2 consumption, RQ values and ICL activity of intact and detached cucumber cotyledons

	Incub. Time (h)	Cotyledon status	Treatment/ Incubation medium	O_2 consumption Rate µmol h^{-1} g dm^{-1}	RQ Value	ICL activity (nmol min^{-1} g^{-1} protein)
A	24 h	Intact	Water	8.2±0.9	1.54	7.4±0.4
	72 h	Intact	Water	168.9±27.0	0.54	19.3±3.9
	72 h	Detached	Water	27.9±8.2	1.04	8.5±2.3
B	72 h	Detached	IAA	27.9±8.8	0.76	6.7±0.6
			2,4-DNP	29.3±4.1	0.84	5.4±0.2
			Kinetin	31.6±1.5	0.78	5.1±0.3
			BAP	24.3±5.7	0.85	4.4±0.3
			GA_3	15.6±1.1	0.80	9.1±0.9
			Ethylene	41.4±0.4	0.87	6.5±0.5
C	72 h	Detached	1% Agar	21.1±7.6	0.78	7.6±1.8
			Filter paper	20.8±0.9	0.84	10.3±1.4
D	72 h	Detached	Weakened testa (Longitudinally)	71.6±12.6	0.70	7.2±0.3
E	72 h	Detached	100% O_2	113.9±44.8	0.67	11.2±2.6
		Detached	Testa removed	124.8±11.4	0.55	13.1±1.3
F	72 h	Intact	Testa removed	228.6±16.4	0.59	19.8±2.1
		Intact	Pure N_2	15.9±5.1	0.98	1.7±0.2

ontogeny and activity of ICL, as well as the lipid mobilization process as a whole.

In contrast, both 2,4-DNP and ARS, uncouplers of oxidative phosphorylation, as well as itaconic acid (IA), a known inhibitor of ICL activity (Jain, 1982), had no effect on germination but either completely inhibited axial growth (2,4-DNP and ARS) or reduced axial growth significantly during the seedling growth phase (IA). This implicated mitochondrial activity only as a prerequisite for post-germinative growth of the axis. Moreover, IA completely inhibited both the *in vivo* and *in vitro* activities of ICL in the cotyledons during the post-germinative growth phase, suggesting that the reduced axial growth observed was a direct result of lipid mobilization inhibition. The latter was confirmed with cycloheximide (CHX) and chloramphenicol (CA). Both these protein synthesis inhibitors effectively inhibited axial growth while CHX also markedly inhibited the *in vivo* activity of ICL in the cotyledons during the post-germinative growth phase. These results suggest that cucumber seeds contained all the necessary proteins to initiate and complete the germination process, but that post-germinative growth clearly depended on the synthesis of new enzyme proteins, including ICL.

The fact that SHAM also completely inhibited axial growth not only emphasized the importance of the alternative electron transport pathway in plant mitochondria for seedling establishment in cucumbers, but emphasized the importance of metabolic events after germination in cucumber. Although not conclusive, especially the results obtained in terms of ICL activity strongly indicate that lipid mobilization in cucumber seed is a post-germinative event which probably dominates cotyledonary metabolism during seedling growth. This corresponds to earlier reports by Chapman and Davies (1983) as well as Bewley and Black (1994) on cucumber but seem to contrast the findings of Penfield et al. (2005) in germinating *Arabidopsis* seed. In the latter seed the authors maintained that lipid breakdown occurs in both the embryo and the endosperm during germination while endospermic lipid reserves require gluconeogenesis and transport of the resulting sugars to the germinating embryo. This suggests that the same sequence of events is not necessarily applicable to oil lipid-rich seed.

Notwithstanding the pivotal role of metabolic events during germination, the developing axis has been postulated in the past to act as a sink in mobilizing respiratory substrate from the cotyledon source (Slack et al., 1977; Davies and Slack, 1981; Chapman and Davies, 1983), one of the objectives of this study. This was tested by replacing the excised axis with artificial sinks (filter paper and a 1% (w/v) agar medium) in order to simulate the possible sink effect of the axis. Besides the artificial filter paper sink that stimulated ICL activity slightly, these manipulation techniques had virtually no effect on either of the measured physiological parameters suggesting that an axial sink effect alone is of small consequence.

In light of early reports on axial hormone involvement in the activation of metabolic events and storage reserve mobilization in cotyledons of germinating squash (Penner and Ashton, 1967), sunflower (Servettaz et al., 1976) and watermelon (Longo et al., 1979) seeds as well as a recent report on ABA involvement in *Arabidopsis* (Penfield et al., 2006), the possibility that storage reserve mobilization in cucumber is under hormonal control was investigated. Incubation of detached cucumber cotyledons in either a crude extract of intact axes or in the presence of intact isolated axes failed to alleviate the effect of axis removal on changes in dry mass, O_2 consumption and RQ values (results not shown). Even separate incubation of detached cotyledons in homogenic solutions of different plant hormones, or in combination, did not alleviate the effect of axis removal on either the respiration rate or ICL activity in the detached cotyledons. Ethylene was the only plant hormone that stimulated O_2 consumption of the detached cotyledons slightly when compared to that of intact cotyledons, but this was not reason enough to believe that lipid mobilization in cucumber cotyledons was under hormonal control.

Further, Chapman and Davies (1983) concluded that no direct evidence exists to substantiate the translocation of a growth substance from the embryonic axis to the cotyledonary tissue as part of a mechanism for controlling enzyme activity. While it can be speculated that the presence of such a growth substance is essential for the development of enzyme activity per se, the origin of such a substance (i.e. cotyledonary or axial) has neither been established nor has a direct control on enzyme activity ever been demonstrated. Sutcliffe and Bryant (1977) also pointed out that it is not appropriate to transfer the hormonal control concept derived from work on cereal grains to dicotyledonous seeds.

The role of the testa in controlling metabolic events in the cotyledons of seeds has also been implicated in the past (Slack et al., 1977). Detached cotyledons with their testae either in place, mechanically weakened or totally removed were compared to intact cotyledons in terms of the effect on metabolic events in the cotyledons. Longitudinal weakening of the testa circumvented the effect of axis removal on respiratory metabolism of the cotyledons to a certain extent, but had no effect on the ICL activity. A combination treatment of replacing the axis with an artificial sink together with weakening of the testa alleviated the axis removal effect by approximately 50%. This indicated that the inhibiting effect of axis removal on the respiratory rate in cotyledons could not be ascribed to the controlling role of the axis only, but that the testa also contributed to the effect.

Crawford (1977, 1978) postulated that the testa is impermeable to O_2, that germinating seeds always experience a period of anaerobiosis during germination and that O_2 becomes available to the cotyledons only after the radicle protrudes the testa. Since both mitochondrial activity and β-oxidation of fatty acids depend on the availability of O_2 the testa might be a limiting factor for these metabolic events to proceed in the cotyledons. This O_2 requirement was clearly demonstrated by incubating intact seeds in the presence of N_2 prior to measuring the respiration rate. Both the ICL activity in and O_2 consumption by intact cotyledons were severely inhibited. Increasing the O_2 concentration in the surrounding atmosphere (100%) increased O_2 consumption by the detached cotyledons by more than 50%. This resulted in a marked decrease in calculated RQ values indicating a major change in metabolism. The increased O_2

availability also stimulated the ICL activity in detached cotyledons to a great extent.

Subsequently, the effect of complete testa removal from detached cotyledons, but without increasing the O_2 concentration in the environment, was investigated. This resulted in almost a complete alleviation of the inhibitory effect axis removal had on O_2 consumption, calculated RQ values and ICL activity. It appeared that when O_2 was made more readily available by removal of the testae from detached cotyledons, the effect of axis removal on the measured metabolic events was totally overcome. Furthermore, removal of the testa from intact seeds at 24 h of incubation increased both O_2 consumption by and ICL activity in the intact cotyledons. Interestingly, cucumber seeds naturally shed their testae between 48 h and 72 h of incubation (Witztum and Gersani, 1975). However, it was shown in this study that detached cotyledons lost this ability and maintenance of a respiration rate equal to that of intact cotyledons could only be achieved when O_2 was made available by mechanically removing the testa.

In conclusion, it is postulated that O_2 availability is probably more important for reserve mobilization to commence in cucumber cotyledons during the post-germinative phase than the axis itself, and shedding of the testa forms part of the controlling mechanism. The fact that the negative effect axis removal had on respiration and ICL activity was greatly overcome by either supplying O_2 or by weakening the testa longitudinally, support this postulate. The possible controlling role of the axis or testa or both on metabolic events in cucumber cotyledons, including key enzyme activities and intermediate metabolite levels as well as cytosolic and organelle protein synthesis activities are currently under further investigation.

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